

The *Src*-suppressed C Kinase Substrate, SSeCKS, Is a Potential Metastasis Inhibitor in Prostate Cancer¹

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ABSTRACT

The molecular mechanisms leading to prostate cancer remain poorly understood, especially concerning the progression to the metastatic form. SSeCKS, a major protein kinase C substrate with tumor suppressor activity, is likely the rodent orthologue of human Gravin/AKAP12, a scaffolding protein for protein kinases A and C. *Gravin* was mapped as a single-copy gene to 6q24-25.2, a hotspot for deletion in advanced prostate cancer, and therefore, we investigated the role of SSeCKS/Gravin in prostate oncogenesis. SSeCKS/Gravin protein was detected in untransformed rat and human prostate epithelial cell lines EP12 and PZ-HPV-7, respectively, and in human prostatic epithelium, especially basal epithelial cells. In contrast, SSeCKS/Gravin protein and RNA levels were severely reduced in human (PC-3, PPC-1, LNCaP, DU145, and TSU) and rat Dunning (AT3.1 and MatLyLu) prostate cancer cell lines. The regulated reexpression of SSeCKS in MatLyLu cells induced filopodia-like projections and a decrease in anchorage-independent growth. In nude mice, SSeCKS reexpression slightly decreased primary-site tumor growth but severely decreased the formation of lung metastases. Primary-site tumors that progressed lost regulated SSeCKS reexpression. SSeCKS/Gravin expression was detected in benign human prostatic lesions and well-differentiated carcinomas but not in undifferentiated lesions with Gleason sums ≥ 6 . Our data suggest a role for the loss of SSeCKS/Gravin in the metastatic progression of human prostate cancer.

INTRODUCTION

Prostatic adenocarcinoma is the most common noncutaneous malignancy in men in the United States, associated with roughly 170,000 new cases and 31,000 deaths in the United States in 2000 (1). Especially hard hit are African-American men, of which the incidence is highest in the world (2). A critical factor in prostate cancer-related mortality is the metastatic potential of the tumor cells and whether the disease will disseminate to secondary sites such as the femur or pelvis. An even larger number of United States men show localized, nondisseminated prostatic cancer with much lower risk of mortality. The high frequency of benign prostatic hyperplasia in men is associated with the continuous growth of the prostate with age. However, the link between benign prostatic hyperplasia, nondisseminated prostatic adenocarcinoma, and MPA³ remains unclear. Much data regarding the molecular biology of prostate cancer development and progression comes from a pedigree of Dunning R-3327 rat prostate cancer lines with varying degrees of tumorigenicity, metastatic potential, and androgen-dependency (3). For example, the G cancer line is poorly tumorigenic in syngeneic Copenhagen rats, whereas MLL cancer cells induce intra- and extraprostatic primary-site tumors and lungs metastases; the AT-3-derived lines are highly tumorigenic and metastatic and also androgen-independent.

The molecular pathogenesis of MPA is a multistep process involving the activation of endogenous oncogenes as well as the loss of tumor suppressor/cancer susceptibility genes. No single oncogene has been associated with MPA, yet $\geq 40\%$ of cases studied contain activating mutations or increased expression of oncogenes such as *ras*, *myc*, or *fos* (4, 5). Even more significant is the loss of critical tumor suppressor functions such as p53, DCC, and p^{Rb}, or cytoskeletal/adhesion molecules such as E-catenin/ α -cadherin (6, 7). These losses correspond to allelic deletions in chromosomes 5q, 17p, 18q, and 13q, found in a small but significant population of MPAs, and deletions in 10q and 6q, found in $>60\%$ of MPAs.

To date, only a handful of metastasis-suppressing genes have been identified, usually by cDNA subtraction and microcell-mediated chromosomal transfer. Examples include nm23 (NME1), KAI1, KiSS1, BrMS1, MKK4/SEK1, CD44, and maspin (reviewed in Ref. 8). Typically, the expression of these gene products is inversely proportional to metastatic potential. The reexpression of these proteins, besides suppressing the production of spontaneous and experimental metastases in animal models, correlates with decreased colony-forming activity in soft agar, motility, and invasiveness into Matrigel and increased cell-cell adhesion and immunosensitivity.

We identified a potential tumor suppressor, SSeCKS, of which the expression is down-regulated in *src*- and *ras*-transformed fibroblasts (9). Reexpression of SSeCKS suppresses *src*-induced oncogenesis such as growth factor- and anchorage-independence, loss of contact inhibition, and metastatic potential, mainly by reorganizing actin-based cytoskeletal architecture (10). SSeCKS is likely the rodent orthologue of human Gravin/AKAP 12, a scaffolding protein for PKC and PKA (11). SSeCKS also plays a role in G₁→S progression by modulating cyclin D expression and by sequestering G₁-phase cyclins in the cytoplasm (12). Here, we show that *gravin* maps to a single chromosomal site, 6q24-25.2, a hotspot for deletion in advanced prostate cancer (13-22). We show that SSeCKS/Gravin expression is abundant in untransformed human and rat prostate cell lines, in normal prostatic epithelial cells, and in undifferentiated human prostate cancers *in vivo* but down-regulated in prostate cancer cell lines and in high-grade cancers *in vivo*. Moreover, reexpression of SSeCKS in MLL cells suppresses cell rounding, anchorage-independent growth, and the generation of secondary lung metastases in nude mice and increases cell-cell adhesion. These data suggest a role for the loss of SSeCKS/Gravin expression in the onset of prostate cancer metastasis.

MATERIALS AND METHODS

Cell Culture. MLL cells, EP12 (EPYP-1; a gift of K. Pienta, University of Michigan Comprehensive Cancer Center, Ann Arbor, MI), LNCaP/tTA (LNGK9; a gift of T. Powell, Memorial-Sloan Kettering Cancer Center, New York, NY; Ref. 23), and HeLa (ATCC CCL2.1) were grown in Dulbecco's modified Eagle's media (DME) (Life Technologies, Inc., Rockville, MD) supplemented with 10% FCS (Life Technologies, Inc.). P69 (P69SV40T), M2182, and M12 (gifts of J. Ware, Medical College of Virginia, Richmond, VA) were grown in RPMI 1640 plus insulin, transferrin, and selenium (Collaborative Biochemicals); dexamethasone (Sigma Chemical Co., St. Louis, MO); and EGF (Collaborative Biochemicals), as described (24).

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³ The abbreviations used are: MPA, malignant prostatic adenocarcinoma; MLL, MatLyLu; tet, tetracycline; IFA, immunofluorescence; FISH, fluorescence *in situ* hybridization; DAPI, 4',6-diamidino-2-phenylindole; PKA, protein kinase A; PKC, protein kinase C.

Production of Tetracycline-regulated MLL Cell Lines. MLL/TAKE cells, expressing a tet-regulated tTA transactivator (25), were produced by transfecting with CaPO₄/DNA precipitates containing 3.5 μ g of pTet-tAK and 0.6 μ g of pRSV/hygro followed by selection of stable transfectants in 400 μ g/ml of hygromycin (Sigma Chemical Co.). Individual clones were tested for the ability to induce expression of tet_r/luciferase (pUHD13-3) in the absence of tet (26). Clones 2 and 7 were chosen for secondary transfection with 3.8 μ g of pUHD10-3/SSeCKS (27) and 1.6 μ g of pBABE/puro (28), and stable transfectants were isolated after selection in hygromycin and puromycin (8 μ g/ml). All of the cells were selected in 5 μ g/ml tet and then maintained on 0.7 μ g/ml.

FISH Analysis. FISH analysis was performed by See DNA Biotech, Inc. (Downsview, Ontario, Canada). A 6.2-kb Gravin cDNA fragment was labeled with Biotin-14-dATP using a Life Technologies, Inc. BioNick kit according to the manufacturer's specifications. Slides were prepared with human lymphocytes grown in α -minimal essential medium containing 10% FCS, phytohemagglutinin, and bromodeoxyuridine (180 μ g/ml; Sigma Chemical Co.), then grown for 6 h in medium containing thymidine (2.5 μ g/ml). FISH detection was performed as described previously (29, 30). Among 100 mitotic figures that were checked, 81 showed signals on one pair of the chromosomes (*i.e.*, 81% hybridization efficiency). DAPI banding patterns mapped the signals to the long arm of chromosome 6, and based on the summary from 10 independent photographs, Gravin was mapped by higher resolution to 6q24-25.2. No additional loci were identified by FISH detection, suggesting the absence of highly conserved gene family members.

Colony Assay in Soft Agar. Cells (10^4) were plated into soft agar in 6-cm wells as described previously (10) and then grown for 3 weeks at 37°C with feedings of fresh medium twice/week.

Northern and Western Blotting. Total or poly(A)-selected RNAs were electrophoresed, blotted, and probed with ³²P-rat SSeCKS cDNA as described (9). Radioimmunoprecipitation assay lysates containing 40–100 μ g of total protein were prepared and immunoblotted using rabbit polyclonal anti-SSeCKS immunoglobulin as described (31), using either alkaline phosphatase- or horseradish peroxidase-labeled secondary antibodies followed by Western Blue substrate (Promega, Madison, WI) or ECL (New England Nuclear, Boston, MA) for visual or chemiluminescence detection, respectively. Images were scanned on an Agfa Duoscan T1200, digitized on a PowerMac G3 (Apple) computer using Adobe Photoshop version 4.01, and quantified using the UN-SCAN-IT Gel program version 4.3 (Silk Scientific, Orem, UT).

IFA and Immunohistochemistry Analyses. Cells seeded onto 22-mm² coverslips were fixed and stained with immunoaffinity-purified rabbit polyclonal anti-SSeCKS (31) or rabbit anti-Gravin sera (32) as described previously (27). Immunocytochemistry was performed as described (32). Slides were viewed on an Olympus IX-70 fluorescent microscope and digitized using a Sony Catseye camera connected to a PowerMac G3 computer. Image analysis was performed using Adobe Photoshop 4.01.

Tumor and Metastasis Formation in Nude Mice. Six-week-old female nude mice (Taconic Farms, Germantown, NY) were injected s.c. with 10^5 MLL/vector or MLL/SSeCKS clones. The viability of the cells was >90% as determined by trypan blue exclusion. All of the mice were fed water containing 100 μ g/ml tet plus 5% sucrose until the primary tumors were palpable (2–4 mm), at which point the tet-water was withdrawn. Mice were sacrificed 3 weeks after injection. The primary tumors were measured and weighed, and the lungs were stained for metastases by injecting India ink (30 ml of ink plus 4 drops of 1 M ammonium hydroxide, diluted into 200 ml of dH₂O) into the trachea for 10 min at room temperature followed by several washes in PBS. Surface metastases, which exclude the dye, were then counted.

RESULTS

Mapping of SSeCKS/Gravin. Rodent SSeCKS and human Gravin/AKAP12 show 83% identity over the first ~1000 amino acid (a.a.), <20% similarity over the next ~500 a.a., and identity in two 15-a.a. stretches at the C-termini, one of which encodes a PKA anchoring site (11). Full-length SSeCKS cDNA recognizes Gravin mRNA under conditions of stringent hybridization (32). Using a Gravin cDNA probe, we mapped *gravin* by FISH to chromosome 6q24-25.2 (Fig. 1). These map coordinates are confirmed by microsatellite markers (Sanger Sequencing Center, Cambridge, United

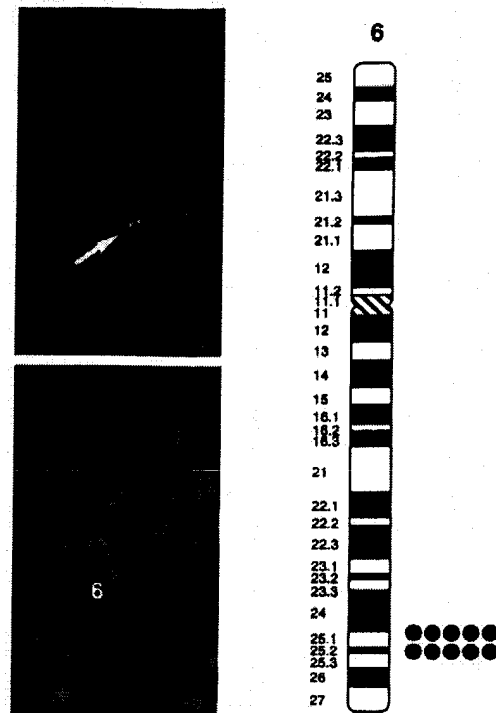


Fig. 1. Mapping of Gravin/AKAP12 to chromosome 6q24-25.2. FISH analysis was performed on metaphase chromosomes of human peripheral blood lymphocytes using a FITC-labeled Gravin cDNA as described in "Materials and Methods." The chromosomes were counterstained with DAPI, and the FITC and DAPI images were overlapped to identify specific chromosomes. Note the lone *gravin* signal (allele pair) at 6q. Of 100 chromosome sets analyzed, 81% showed hybridization signals, and 10/10 randomly chosen sets showed FITC signals corresponding to region 6q24-25.2 (● next to right panel).

Kingdom). Secondary hybridization signals were not detected, which might reflect a second family member. FISH analysis using a full-length SSeCKS cDNA probe identified the same, singular region (data not shown). Moreover, mouse SSeCKS maps to the Tsga12 locus at the centromeric end of chromosome 10p, which is syntenic with human chromosome 6q24-27⁴, strongly suggesting that SSeCKS and Gravin/AKAP12 are orthologues. Deletions in this region are associated with advanced, nonorgan confined prostate cancer cases (13–22), suggesting a possible role for SSeCKS/Gravin in prostate oncogenesis.

Loss of SSeCKS/Gravin Expression in Human and Rat Prostate Cancer Cell Lines. Northern blots containing total or poly(A)-selected RNA from Dunning rat prostate cancer cell lines or tumors (grown in Copenhagen rats) or from human prostate cancer cell lines were probed under stringent conditions with rat SSeCKS cDNA. Fig. 2A shows a progressive loss of SSeCKS RNA signal in MLL and AT-3.1 cancer cells (5- and >20-fold lower, respectively, as defined by densitometry) compared with the mildly transformed G cell line. AT-3.1 cells, which are androgen-independent, are slightly more metastatic in nude mice than MLL (3). SSeCKS RNA levels in R3327-H-, MatLu-, MLL-, or AT-3-induced tumors are reduced >5-fold compared with levels in the G cell line. Fig. 2B shows a similar reduction in Gravin/AKAP12 RNA levels in various human prostate cancer cell lines compared with normal human prostate. In addition to the expected 6.5-kb *gravin* message, PC-3 and PPC-1 cells displayed an additional RNA transcript (~8.5 kb) that hybridized to the rat SSeCKS probe. Growth of the androgen-responsive LNCaP cells (33) in the absence of androgens for >9 months had no effect on Gravin/

⁴ Internet address: <http://www.informatics.jax.org>.

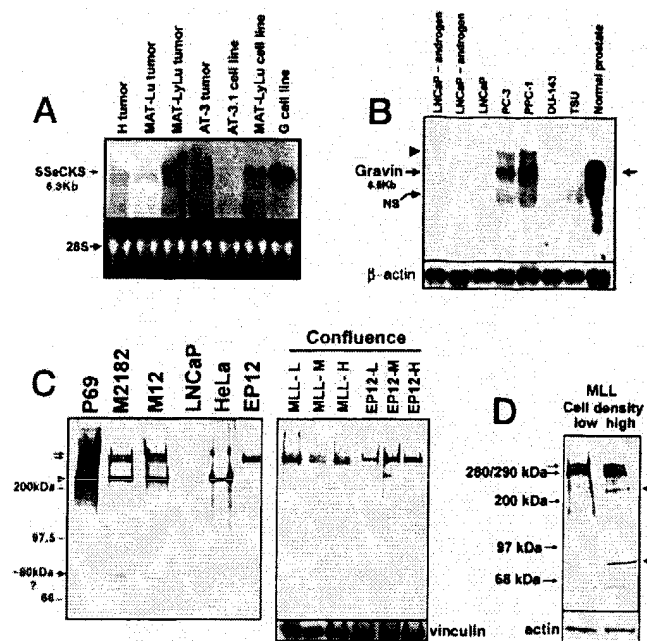


Fig. 2. Loss of SSeCKS/Gravin expression in prostate cancer. *A*, Northern blot analysis of total RNA (25 μ g/lane) from Dunning rat prostate cancer cell lines grown in culture or in Copenhagen rats, probed with 32 P-rat SSeCKS cDNA (SSeCKS message is 6.3 kb). Stained with ethidium bromide, 28S rRNA is the loading control. The R3227-G cell line, derived from the original R3227 tumor (reviewed in Ref. 45), is strictly androgen-dependent and displays poor transformed growth potential *in vitro*. The R3227-H tumor has cells with mixed tumorigenic potential; Mat-Ly-Lu and Mat-Lu were derived clonally from AT-1, an isolated tumorigenic clone from R3227-H, and AT-3 is a highly metastatic, anaplastic clone derived from lines grown in castrated Copenhagen rats. *B*, Northern blot analysis of poly(A)⁺ RNA (2 μ g/lane) from human prostate cancer cell lines or total normal human prostate, probed at high stringency with 32 P-rat SSeCKS cDNA (Gravin/AKAP12 message is 6.5 kb; NS, nonspecific signal; upper band with \blacktriangleright identifies an aberrant \sim 8.5 kb Gravin message in PC-3 and PPC-1 cells). LNCaP, androgen: two LNCaP cells lines grown without androgens for >9 months. The blot was stripped and reprobed for β -actin as a loading control (below). *C*, Western blot of protein (50 μ g/lane) from various rodent or human prostate cell lines, probed with polyclonal anti-SSeCKS immunoglobulin. The blot was stained with Amido Black (Bio-Rad) to control for protein loading (data not shown). Confluency: L, low ($<40\%$); M, medium (70–80%); H, high (saturation density). SSeCKS isoforms are identified by arrows relative to protein molecular weight markers (M_r 290,000/280,000, doublet arrow and M_r 240,000, \blacktriangleright). Densitometry indicated that the relative levels of SSeCKS in EP12 was 4- and 10-fold higher in M and H cultures, respectively, relative to L cultures, whereas the relative SSeCKS levels in MLL M and H cultures was 1.5-fold lower than in L cultures. After stripping, the blot was reprobed with anti-vinculin as a loading control. *D*, high culture density of MLL does not alter the relative abundance of the M_r 290,000/280,000 SSeCKS isoforms but increases the abundance of the $M_r \sim 80,000$ isoform. Lysates were probed with anti-actin as a loading control. The relative abundance of SSeCKS/Gravin protein levels was determined by densitometry.

AKAP12 transcript levels, possibly because LNCaP cells contain a 6q24-ter deletion (34), a region encompassing *gravin* (Fig. 1). Thus, the loss of SSeCKS/Gravin message is typical in prostate cancer lines.

We then compared the relative levels of SSeCKS/Gravin proteins in various untransformed and cancerous prostate cell lines. In most fibroblastic and epithelial cells, SSeCKS was shown to be expressed as several isoforms: M_r 290,000 (myristylated), M_r 280,000 (nonmyristylated; contains a novel NH₂-terminal 7 a.a. resulting from alternative splicing), M_r 240,000 (a proteolytic fragment of M_r 290,000/280,000 lacking an NH₂-terminal domain), and M_r 43,000 (an internal proteolytic fragment). The typical Gravin/AKAP12 isoforms are M_r 305,000, 287,000, 250,000 and 43,000, where the larger sizes of the former three results from an extra COOH-terminal 100 a.a. in Gravin/AKAP12 compared with rodent SSeCKS. These isoforms were confirmed by immunoblotting using several sets of polyclonal and monoclonal antibodies, as well as by mapping with genetic mutants (32).

The P69 series (24) consists of human prostate epithelial lines with increasing oncogenic characteristics in nude mice: P69 are nontumori-

genic cells immortalized with SV40 T_{ag}; M2182 are nonmetastatic variants that form tumors at the primary injection site; and M12 are variants selected in nude mice that form lung metastases after i.p. or intraprostatic injection. Fig. 2C shows a severe decrease (>10 -fold) in the levels of the M_r 305,000/287,000 major Gravin isoforms in both M2182 and M12 cells compared with the parental P69 cells.

Our previous data indicated that the level of SSeCKS RNA and protein increases dramatically under contact-inhibited growth conditions (9, 12, 31, 35). Fig. 2C shows a >10 -fold increase in SSeCKS protein levels in rat EP12 cultures in transition from subconfluence (L) to several days of confluent growth (H), as determined by densitometry. In contrast, SSeCKS levels in MLL cells, which are 4-fold lower than EP12 cells, are not affected by culture density. However, the abundance of the M_r 240,000 SSeCKS isoform as well as a novel $M_r \sim 80,000$ isoform increases in MLL after growth at high density (Fig. 2D). The M_r 240,000 isoform (M_r 250,000 in human cells) is a proteolytic cleavage product lacking NH₂-terminal sequences (32). Thus, SSeCKS/Gravin protein levels are induced in both epithelial cells and fibroblasts in response to increased culture density, and this induction mechanism is inactive in prostate cancer cells.

Tet-regulated SSeCKS Reexpression Suppresses MLL-induced Oncogenesis. We demonstrated previously that the tet-regulated reexpression of SSeCKS led to the suppression of *src*-induced oncogenic growth fibroblasts (10). In that study, reexpression of SSeCKS to levels 2–25-fold over those in untransformed parental cells led to the selective loss of *src*-induced oncogenic growth parameters such as anchorage- and growth-factor independence and Matrigel invasiveness but did not inhibit cell proliferation, indicating that the SSeCKS effects were not simply attributable to toxicity. Because MLL cells encode at least one activated

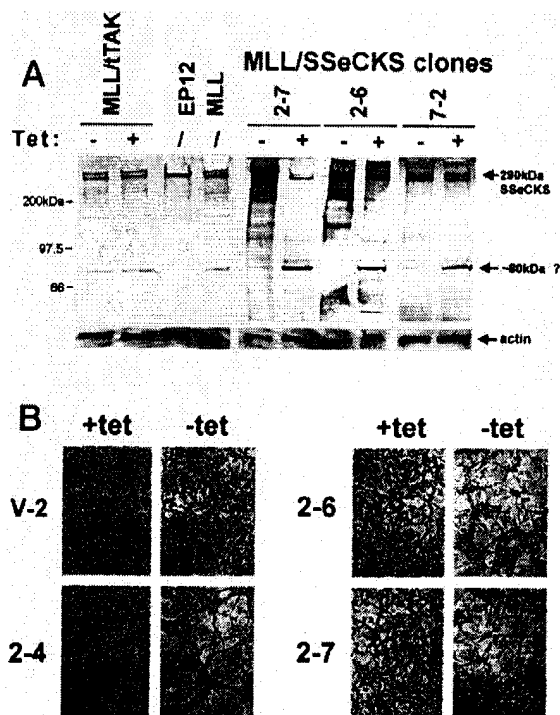
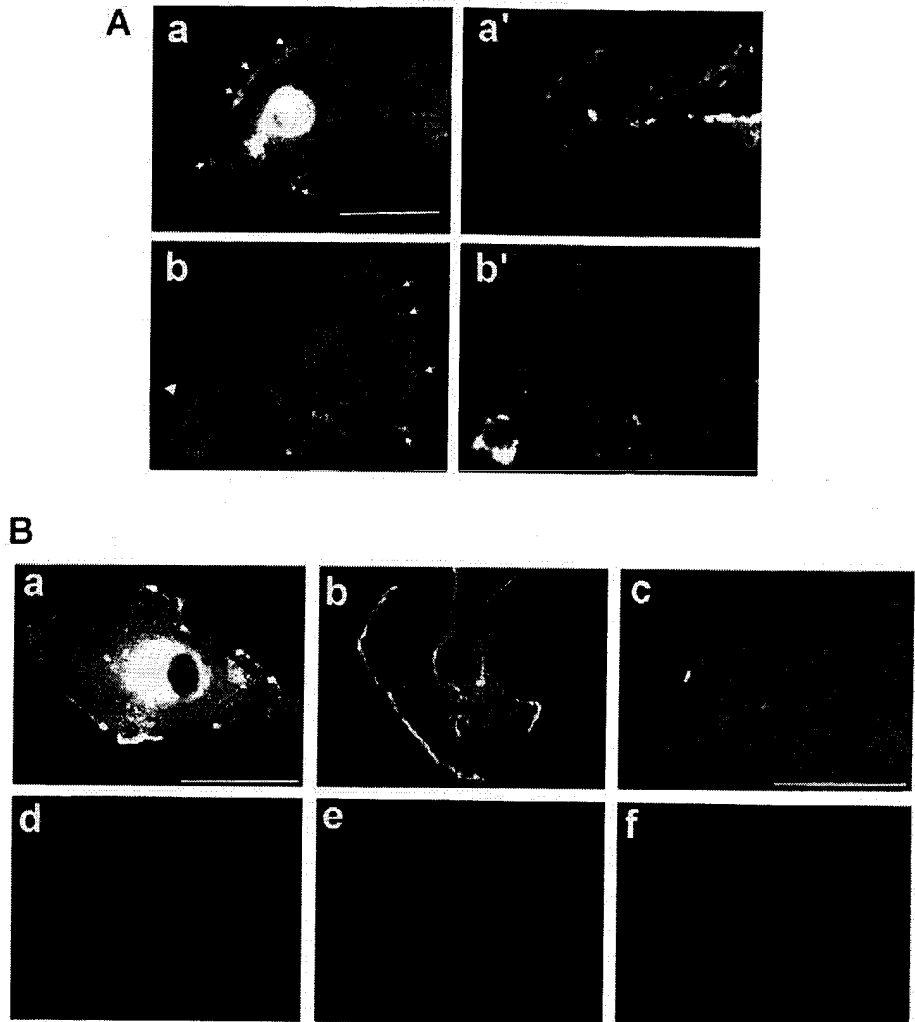


Fig. 3. Production of MLL cell lines with tet-regulated SSeCKS expression. *A*, 50 μ g of protein from MLL/TAK, EP12, MLL, or MLL/SSeCKS clones 2-6, 2-7, and 7-2 were immunoblotted for SSeCKS. The M_r 290,000 and $M_r \sim 80,000$ SSeCKS isoforms, arrows (right), relative to protein markers (left). Lysates were probed with anti-actin as a loading control. *B*, reexpression of SSeCKS suppresses morphological transformation and increases cell-cell adhesion. Phase contrast microscopy of MLL[tet/vector] (V-2) or MLL[tet/SSeCKS] clones (2-4, 2-6, and 2-7) grown in the presence or absence of tet for 3 days. Note the decreased refractility and increased cell flattening in the MLL[tet/SSeCKS] clones grown without tet. $\times 100$.

Fig. 4. IFA analysis of SSeCKS and F-actin. *A*, EP12 cultures fixed in acetone/formaldehyde were stained for SSeCKS (*a*, *b*) or F-actin (*a'*, *b'*; TRITC-phalloidin). Note the cortical cytoskeletal SSeCKS staining marked by enrichments in the perinuclear region, in membrane ruffles (*a*, arrows), in focal contacts at the lagging edge (*b*, Δ), and in actin-rich concentrations at the leading edge (*b*, arrows). *B*, reexpression of SSeCKS in MLL-[tet/SSeCKS] cells grown without tet (*a* and *b*) shows enrichment of SSeCKS in membrane ruffles (*a*) and in lamellipodia (*b*). EP12 (*c*), showing typical cell-cell adhesion and epithelial morphology, is shown for comparison. In contrast, little endogenous SSeCKS staining is detected in MLL-[tet/vector] cells grown with (*d*) or without (*e*) tet or MLL[tet/SSeCKS] cells grown with tet (*f*). $\times 630$; size bars, 20 μm .



Ha-ras allele (4), we addressed whether SSeCKS could suppress *ras*-associated tumorigenic growth by producing MLL cells with tet-regulated SSeCKS expression. Fig. 3*A* shows similar levels of SSeCKS protein in the MLL/tAK cells compared with the parental MLL cells. The MLL/tAK cells express a tet-regulated form of the tTA transactivator (25), which we found to be much less toxic than the constitutively expressed tTA. Expression of the *M*, 290,000 SSeCKS isoform was induced 4–20-fold in several independently derived clones grown without tet (Fig. 3*A*). These clones also express background levels of SSeCKS in the presence of tet.

The MLL[tet/SSeCKS] clones exhibited increased cell flattening, decreased refractility, and increased cell-cell interaction after the removal of tet (Fig. 3*B*). SSeCKS induced a fibroblast-like morphology (Fig. 3*B*) rather than the epithelial morphology typified by EP12 cells (Fig. 4*B*, *c*). tAK caused mild cell flattening (Fig. 3*B*), and although this was always significantly less than that induced by SSeCKS, it is impossible to exclude that SSeCKS and tAK work cooperatively in this regard. In contrast, increased cell-cell adhesion was induced by SSeCKS reexpression and not by tAK.

We analyzed the compartmentalization of SSeCKS by IFA using immunoaffinity-purified anti-SSeCKS immunoglobulin (31). Fig. 4*A* shows enrichment of SSeCKS in the perinuclear regions of EP12 cells as well as a cortical cytoskeletal distribution. SSeCKS was enriched in actin-dense membrane ruffles (Fig. 4*A*, *a* and *a'*; arrows), in focal

complexes connected to actin stress fibers (Fig. 4*A*, *b* and *b'*; Δ), and in actin-dense bundles at the lagging edge (Fig. 4*A*, *b*, *b'*; arrows). In MLL[tet/SSeCKS] cells grown without tet, reexpressed SSeCKS was enriched in membrane ruffles (Fig. 4*B*, *a*) and in lamellipodia (Fig. 4*B*, *b*).

We then addressed whether SSeCKS expression affected parameters of *in vitro* oncogenic growth. Fig. 5*A* shows that expression of tAK alone (V-2 or V-7) was somewhat inhibitory to proliferation in medium with 10% serum, probably attributable to squelching of transcription factors by the VP16 moiety of tAK (10, 27, 36). Although SSeCKS expression in some clones decreased proliferation rates slightly more than vector controls, it is impossible to separate SSeCKS- and tAK-induced effects. However, fluorescence-activated cell-sorted MLL cells transiently cotransfected with SSeCKS and Green Fluorescent Protein expression vectors showed no significant changes in proliferation rates compared with Green Fluorescent Protein controls (data not shown). Thus, we conclude that SSeCKS reexpression does not significantly alter the proliferation rate of MLL cells.

Fig. 5*B* shows that SSeCKS expression inhibited anchorage-independent growth 4–5-fold over that induced by tAK alone. The ~40% decrease in colony-forming activity in vector control cells probably relates to the inhibitory effects of tAK. We sought to determine whether SSeCKS could inhibit the anchorage-independent

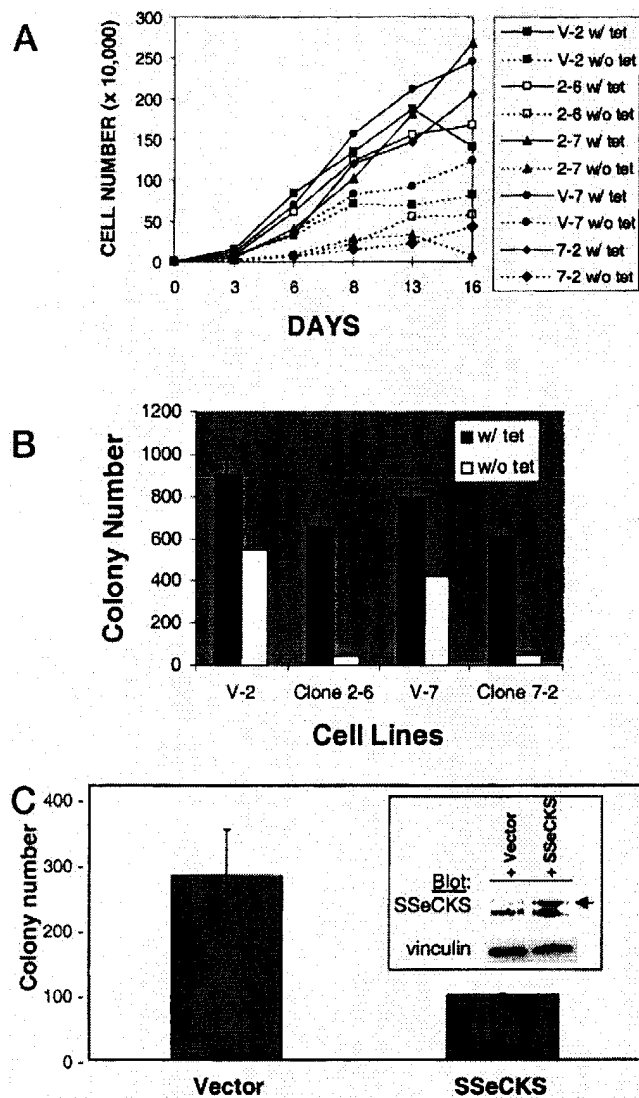


Fig. 5. Ectopic SSeCKS decreases MLL proliferation rate and anchorage-independence. **A**, MLL[tet/vector] control (V-2, V-7) or MLL[tet/SSeCKS] clones were grown in medium containing 10% FCS with (—) or without tet (---) and then monitored for proliferation. All of the experiments were performed in triplicate, and variations at any time point for a given sample were <15%. **B**, triplicate aliquots of 10^4 MLL[tet/vector] or MLL[tet/SSeCKS] cells were grown in soft agar overlays for 3 weeks at which point the number of colonies formed was determined. In the absence of tet, control cells formed ~40% fewer colonies than if grown with tet. In contrast, the MLL[tet/SSeCKS] clones produced 8–12-fold fewer colonies in the absence of tet. Several SSeCKS-expressor soft-agar colonies were isolated and grown in culture, and all showed a loss of tet-inducible SSeCKS expression (data not shown). **C**, SSeCKS-induced suppression of anchorage-independence in the absence of tTAK. Triplicate aliquots of MLL cells were transiently transfected with a 1:4 ratio of pBABEhygro and either pcDNA3 or pcDNA/SSeCKS, and then grown for 3 weeks in soft agar supplemented with hygromycin. Transfection efficiencies were normalized by cotransfecting 1.5 μ g of pEGFP and monitoring cell fluorescence.

growth of MLL in the absence of the tet system. Fig. 5C shows that transient expression of SSeCKS decreased the colony-forming efficiency of MLL ~3-fold compared with vector controls. Thus, the suppressive effects of SSeCKS on anchorage-independent growth are separable from those of tTAK.

The SSeCKS/Gravin M_r ~80,000 Isoform: A Marker for Prostate Cancer? We noted that the M_r ~80,000 isoform was present in the MLL/tTAK cells and in all of the MLL[tet/SSeCKS] clones grown with tet, whereas tet removal correlated with a 2–10-fold decrease in the abundance of this isoform. This isoform was also detected in the

human tumorigenic lines M2182 and M12 but not in untransformed parental P69 prostate epithelial cells (Fig. 2C; arrow). We investigated whether the M_r ~80,000 isoform was produced by a factor present in either prostate cancer or untransformed cells. NP-40 lysates from MLL and EP12 cells were mixed at a 1:1 ratio to determine whether factors from one lysate would decrease the presence of the M_r ~80,000 isoform. Table 1 shows that mixing of the lysates at 30°C for 5 min caused a decrease in the abundance of the M_r ~80,000 isoform (compared with incubation of the MLL lysate alone). Preboiling of the MLL lysate did not effect the EP12-induced decrease, whereas boiling of the EP12 lysate did. This indicates that EP12 encodes a heat-labile factor that is antagonistic to the production or stability of the M_r ~80,000 isoform.

Suppression of *in Vivo* MLL Metastasis by SSeCKS. We addressed whether SSeCKS expression could inhibit either growth of primary tumors or generation of secondary lung metastases. Nude mice were injected in their flanks with 10^5 cells and then maintained on tet in their drinking water until tumors were palpable (2–4 mm), whereupon the tet-water was removed. Several independently derived vector control and SSeCKS reexpressor clones were compared in this analysis to increase significance. SSeCKS expression only mildly inhibited tumor growth at the primary site in comparison with vector controls. The small effect (20% reduction) was 8–10 days after initial tumor palpation, but subsequently, there was no significant difference in tumor size or doubling rates between SSeCKS and control tumors (Fig. 6A). However, analysis of progressing primary-site MLL[tet/SSeCKS] tumors revealed a loss of tet-regulated SSeCKS expression (Fig. 6C), suggesting that the progressors were variants that had defeated the inducible expression of SSeCKS. Growth of cells *in vitro* from these primary tumors (in DME + hygromycin/puromycin) showed a lack of inducible SSeCKS in the absence of tet (data not shown). These data indicate that SSeCKS reexpression did not significantly decrease the formation or growth rate of primary-site tumors.

In contrast, mice receiving the MLL[tet/SSeCKS] cells in the absence of tet contained far fewer lung metastases 3 weeks after primary tumor cell injection than the vector controls (Table 2; Fig. 6B). The inhibition occurred with two independent clones with varying degrees of inducible SSeCKS expression (2–6 and 7–2). Because expression of SSeCKS had no effect on cell motility in a monolayer wounding assay (Fig. 6D), it is unlikely that these cells are defective for cell motility *in vivo*. These data indicate that even mild reexpression levels of SSeCKS, as in clone 7–2, are sufficient to suppress metastatic potential in MLL cells.

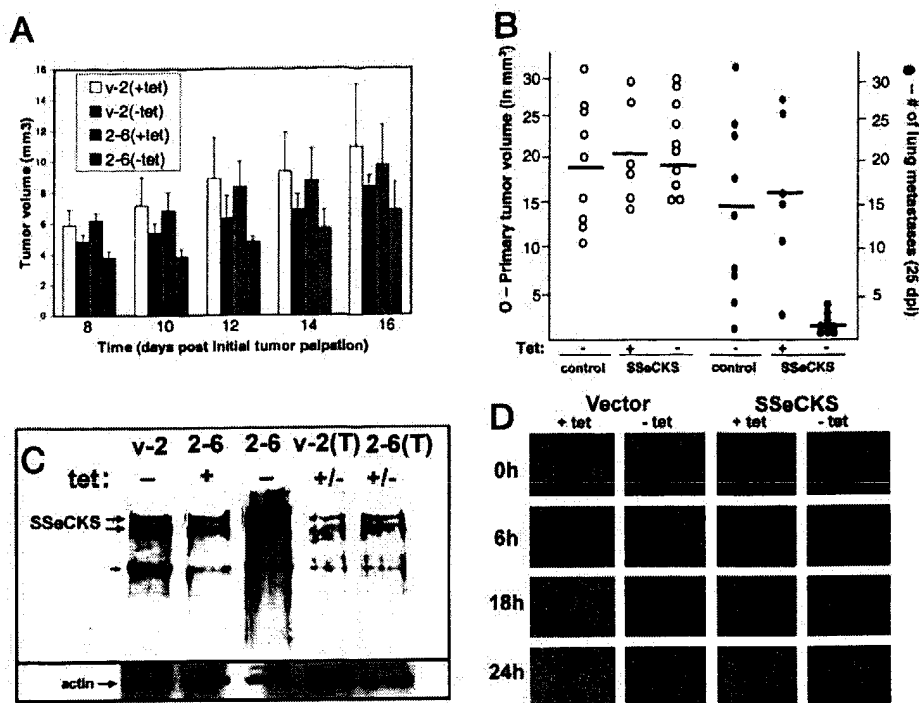
Loss of SSeCKS/Gravin Expression in Well-differentiated Human Prostate Cancer. We analyzed various human prostate lesions for SSeCKS/Gravin expression. SSeCKS/Gravin stained extensively in prostatic epithelial cells, especially the basal epithelial cells, although cell surface staining was detected in some columnar epithelial cells (Fig. 7a). Abundant SSeCKS/Gravin staining was detected in benign prostatic hyperplastic lesions (Fig. 7b) and in well-differentiated carcinomas (Fig. 7c). In contrast, SSeCKS/Gravin staining was absent in undifferentiated carcinomas (Fig. 7d). Noncancerous ducts in the same prostate contain epithelia with robust SSeCKS/Gravin staining. A larger survey of human samples showed a consistent loss

Table 1 Effect of lysate mixing on the generation of the M_r 80,000 SSeCKS isoform

Reactants ^a	Presence of M_r 80,000 band	Length of incubation
MLL lysate + EP12 lysate	—	5 min
MLL lysate (boiled) + EP12 lysate	—	5 min
MLL lysate + EP12 lysate (boiled)	+	>30 min

^a MLL and EP12 lysates were added at a 1:1 ratio.

Fig. 6. Effect of ectopic SSECKS expression on tumor growth in nude mice. **A**, athymic *nu/nu* mice (six/experiment) were injected *s.c.* with 10^5 MLL[tet/vector] or MLL[tet/SSECKS] cells. All of the mice were maintained on tet/sucrose-water (changed every 2 days). When primary tumors were palpable, half the mice were switched to plain water ($-tet$) whereas the others continued with tet-water ($+tet$). The average tumor volumes at primary injection sites are shown; bars, range of tumor volumes in a given cohort. This experiment was repeated twice with two MLL[tet/vector] and two MLL[tet/SSECKS] clones. The *X* axis represents the days after initial tumor palpation. **B**, relative tumor burden at the primary injection site (\circ) compared with lung metastases (\bullet). Results compare two independent experiments of MLL[tet/vector] (clones V-2 and V-7) in mice receiving no tet to "SSECKS," MLL[tet/SSECKS] clones 2-6 and 7-2, in mice receiving tet ($+tet$) or regular water ($-tet$). **C**, eighteen-day tumors from mice in the experiment in **A** were excised and analyzed by Western blotting for SSECKS. Note the loss of tet-regulated SSECKS expression in MLL[tet/SSECKS] clone 2-6 from mice initially fed tet and then fed water lacking tet ($+/-$), compared with 2-6 cells grown in culture. **D**, monolayer wounding assay showing no effect of SSECKS expression on cell motility (clone 2-6) compared with vector (*v-2*) controls.



of SSECKS/Gravin expression in MPAs with Gleason sums >6 (Table 3). MPAs were all positive for prostatic acid phosphatase as a control for stainability. These data correlate the loss of SSECKS/Gravin with the onset of aggressive prostate oncogenesis in humans.

DISCUSSION

We present evidence that the loss of expression of SSECKS/Gravin correlates with increased oncogenic behavior of prostate cancer in both rodents and humans and that SSECKS reexpression suppresses anchorage-independent and metastatic growth. Preliminary evidence indicates a selective loss of SSECKS/Gravin staining in advanced, undifferentiated human prostate cancers with Gleason sums ≥ 6 . These data strongly suggest that the loss of SSECKS/Gravin contributes to the progression of prostate cancer and that SSECKS/Gravin encode tumor suppressor activity.

Relative to untransformed control epithelial cell lines or normal prostate tissue, SSECKS/Gravin RNA and protein levels are markedly reduced in rat and human prostate cancer cell lines or rat prostate tumors grown in syngeneic animals. There was no consistent gradation of SSECKS/Gravin down-regulation correlating with tumorigenic or metastatic phenotype between the cancer cell lines. For example, LNCaP cells had no detectable levels of Gravin RNA, yet these cells

are less tumorigenic in nude mice than PC-3 cells, which had down-regulated, yet detectable levels of Gravin transcript. The P69 series showed a similar down-regulation of SSECKS/Gravin in the tumorigenic M2182 and metastatic M12 variants. However, these clones were selected for spontaneous oncogenic growth in nude mice, and as such, do not reflect the biology of prostate cancer progression. For example, although SSECKS/Gravin RNA levels are decreased in M2182 and M12, they retain chromosome 6q. Indeed, the chromosomal deletions and amplifications in M12 cells are more typical of lung metastases from nonprostate cancers than bony metastases from prostate cancer (24). On the basis of the ability of SSECKS to inhibit formation of MLL-induced lung metastases (below), we would have expected an additional decrease in relative SSECKS/Gravin levels in M12 versus M2182 cells. However, it is most likely that the metastatic potency of our M12 cells has been decreased by repeated passage in culture, because *s.c.* injection of 10^6 M12 cells induced robust primary-site growth but no lung metastases (data not shown).

As in the case of SSECKS reexpression in *src*-transformed NIH3T3 fibroblasts (10), SSECKS reexpression in MLL cells inhibited anchorage-independent growth. We also show that SSECKS could inhibit MLL colony-forming activity in the absence of the tet-regulated system. SSECKS expression itself was not significantly more inhibitory to proliferation than tTAK. However, SSECKS reexpression in the absence of the tet system clearly indicates that SSECKS does not inhibit MLL cell proliferation on its own. Thus, it is unlikely that the decreased colony-forming activity detected after SSECKS reexpression in MLL cells is attributable to inhibition of proliferation by SSECKS. Although SSECKS induced more cell flattening than tTAK alone, we cannot rule out that these effects result from synergy between SSECKS and tTAK. In contrast, SSECKS but not tTAK, could induce cell-cell adhesion in the MLL system.

SSECKS expression antagonizes MLL-induced oncogenesis *in vivo*, especially the generation of lung metastases. Preliminary data indicate that some MLL[tet/SSECKS] cells metastasize to the lungs, based on the ability to select for rare (<25 cells/lung) puromycin/

Table 2. Effect of ectopic SSECKS expression on lung metastasis formation

Clone injected ^a	No. of lung metastases ^b	Size of metastases ^c
V-2	32, 6, 3	4 mm \pm 1
V-7	18, 11, 6	4 mm \pm 1
2-6-11 ^d	0, 1, 0	1 mm \pm 0.5
7-8-14 ^d	0, 0, 0	0
2-6-5 ^d	2, 0, 0	1 mm \pm 0.5

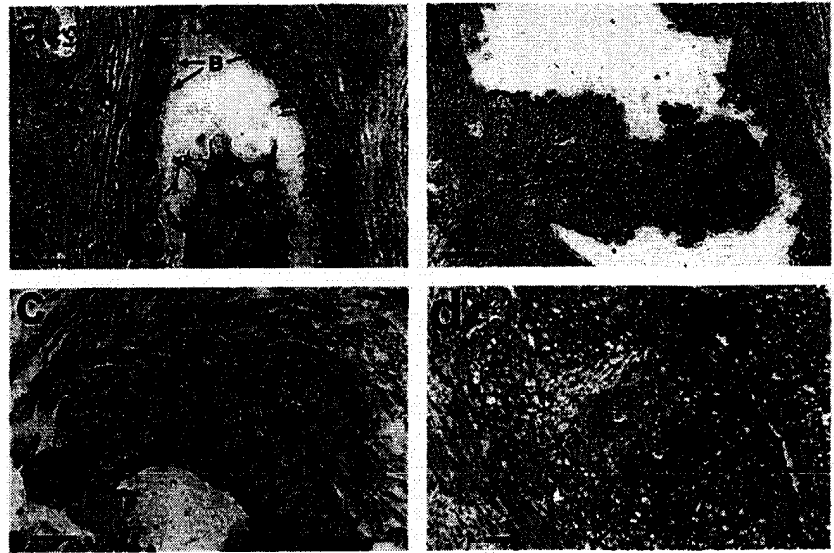
^a 10^5 cells ($>90\%$ viability) injected *s.c.* into flanks.

^b Lung metastases were analyzed 3 wks after initial appearance of primary tumors.

^c Metastases on the surface of lungs were identified by exclusion of India ink staining as described in "Materials and Methods."

^d Subclones of MLL[tet/SSECKS] lines 2-6 and 7-8 isolated by single-cell cloning methods were identified, which had $>95\%$ of cells overexpressing SSECKS ($-tet$) using IFA analysis (data not shown).

Fig. 7. Loss of SSeCKS/Gravin in advanced human prostate cancer *in situ*. Formalin-fixed sections of human prostate representing normal tissue (a) showing enriched Gravin staining in basal epithelial cells (b), benign prostatic hyperplasia (b), well-differentiated prostate cancer (c) and advanced, undifferentiated prostate cancer *in situ* (d) were processed and stained by immunohistochemistry for SSeCKS/Gravin protein as described (31). d is shown at a lower magnification to demonstrate the loss of Gravin staining in a high-grade lesion adjacent to abundant staining in unaffected epithelium; size bars, 10 μ m.



hygromycin-resistant cells in the lungs after collagenase treatment (data not shown). Taken together with our demonstration that SSeCKS reexpression does not decrease MLL cell motility, these data suggest that SSeCKS induces dormancy of metastatic cells in the lung.

The case at which the MLL cells override the tet-regulated SSeCKS expression *in vivo* probably belies the plasticity of these tumor cells. Nonetheless, our results suggest that either the sustained expression of SSeCKS or its repeated dosage in prostate tumors would decrease primary tumor growth and metastatic potential.

The association of SSeCKS with cortical, actin-based cytoskeletal structures (27) leads us to believe that the tumor-suppressive activity of SSeCKS is a direct result of its ability to reorganize cytoskeletal architecture and, subsequently, to reestablish feedback control of signaling pathways. Tumor cells often have normal steady-state levels of major cytoskeletal proteins such as actin and tubulin yet have increased turnover of their polymerized, filamentous forms compared with untransformed cells (37). In contrast, cancer cells have decreased levels of minor or

intermediate cytoskeletal proteins such as vinculin, α -actinin, tropomyosin, or cytokeratins (38). Many groups have shown that reexpression of any one of these latter proteins is sufficient to induce a cytoskeletal reorganization that stabilizes microtubule and microfilament structures. SSeCKS seems to function in a similar manner.

Taken together with the putative scaffolding role for PKA, PKC, and cyclin D (11, 12) in SSeCKS, as well as preliminary data suggesting a role for these scaffolding functions in the control of cyclin D1 expression and $G_1 \rightarrow S$ progression (12), we suggest that SSeCKS controls tumorigenic and metastatic growth by acting as a gating function for signaling and cytoskeletal pathways. Indeed, a paradoxical effect of SSeCKS overexpression is a decrease in apoptosis and anoikis (27), suggesting that SSeCKS suppresses oncogenic pathways leading to caspase activation.

We also noted that SSeCKS expression induced increased cell-cell adhesion in most of the MLL[tet/SSeCKS] clones. E-cadherin expression is lost in MLL (39, 40), possibly because of deletion of both alleles (41). We could not detect E-cadherin or α -catenin in MLL[tet/SSeCKS] cells even after ectopic SSeCKS expression; β -catenin, which is detectable in parental MLL cells, was not affected by SSeCKS (data not shown). A pan-cadherin polyclonal antibody (Sigma Chemical Co.; Ref. 42) to E-, EP-, N-, P, L-, and V-cadherins failed to show any increase in cadherin levels after SSeCKS expression. Thus, cell-cell adhesion may be facilitated by atypical cadherins or by noncadherin moieties such as selectins or integrins. The latter notion is strengthened by data that MLL cells show increased levels of β_1 and α_5 integrins as well as fibronectin (40), which might antagonize mechanisms of normal cell-cell adherence.

An $M_r \sim 80,000$ SSeCKS/Gravin isoform was detected in human and rat prostate cancer cells but not their untransformed control lines or in SSeCKS reexpressors. The likelihood that this is an SSeCKS isoform rather than an induced cross-reactive protein is based on its being recognized by two different monoclonal antibodies raised to rat SSeCKS protein (data not shown). We postulate that untransformed prostate epithelia express a heat-labile factor which either degrades the $M_r \sim 80,000$ isoform or inhibits a tumor-specific protease that generates the $M_r \sim 80,000$ isoform. This protease is unlikely a caspase, because SSeCKS/Gravin lacks any of the three recognition motifs (43). It is interesting to speculate that the $M_r \sim 80,000$ fragment either plays a positive role in tumorigenesis or acts as a dominant-interfering isoform against full-length SSeCKS/Gravin much in the

Table 3 Loss of Gravin expression in malignant prostate cancer

Tissue	Gleason sum	SSeCKS/Gravin	PAP ^a
normal prostate -1		+++	+
normal prostate +2		+++	+
BPH ^c -1		+++	+
BPH -2		++	+
BPH -3		+++	+
BPH -4		+++	+
BPH -5		+++	+
BPH -6		++	+
BPH -7		+++	+
BPH -8		++	+
BPH -9		+++	+
BPH -10		+++	+
CaP ^d -1	4	++	+
CaP -2	4	++	+
CaP -3	5	+/- ^b	+
CaP -4	6	+/-	+
CaP -5	6	+/-	+
CaP -6	7	-	+
CaP -7	9	-	+
CaP -8	9	-	+
CaP -9	9	-	+
CaP -10	9	-	+
CaP -11	10	-	+

^a PAP, Prostatic acid phosphatase.

^b Staining in neoplastic regions; normal ducts in the same samples displayed typical epithelial cell staining.

^c BPH, benign prostatic hyperplasia.

^d CaP, prostate cancer.

same way FAK-related nonkinase (FRNK) antagonizes focal adhesion kinase (FAK) function (reviewed in Ref. 44). If the former role is correct, then antagonists of the putative SSeCKS protease might inhibit parameters of prostate tumorigenesis. The latter role might explain why cells such as MLL have metastatic potential although they express limited levels of SSeCKS. Thus, SSeCKS/Gravin functions might be lost by either chromosomal deletion, transcriptional down-regulation, proteolytic cleavage, or hyperphosphorylation, as we showed in the case of *ras*-transformed Rat-6 cells (31).

In summary, we have demonstrated that the loss of SSeCKS/Gravin expression is typical in prostate cancer cell lines and in human MPA. We also show that SSeCKS/Gravin can suppress prostate oncogenesis *in vitro* and *in vivo*, suggesting that reexpression would antagonize progression of prostate cancer in patients.

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